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14. ABSTRACT The diagnosis of eosinophilic esophagitis (EoE) is based on the presence of $\geq 15$ eosinophils/hpf in the esophagus of a patient with symptoms of esophageal dysfunction in whom GERD is excluded. EoE is likely mediated by interaction of environmental allergens (such as foods) with several genes. Food antigens play an essential role in EoE since specific food elimination diets and amino acid formulas are successful EoE therapy in 60-98% of subjects. Indeed, the majority of children with EoE have specific IgE to foods but they often continue to ingest these foods due to lack of immediate hypersensitivity reactions. This study focuses on the gene-environment interaction of food consumption in food sensitized children with EoE and TGFb1 gene polymorphisms. We hypothesize that in EoE there is a gene polymorphism (TGFb1) environment (food) interaction that contributes to increased IgE mediated TGFb1 expression in the esophagus and increased esophageal remodeling in a subset of EoE subjects. As esophageal stricture formation is an important complication of remodeling in EoE (6-12% of children; 33% of adults), identifying genetic polymorphisms in TGFb1 in EoE may allow the early identification of food sensitized children at risk for the development of this significant complication of EoE.					
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**Introduction:**

The diagnosis of eosinophilic esophagitis (EoE) is based on the presence of  $\geq 15$  eosinophils/hpf in the esophagus of a patient with symptoms of esophageal dysfunction (i.e. dysphagia, anorexia, early satiety, failure to thrive) especially when there is a lack of response to proton pump inhibitor treatment. The prevalence of EoE is approximately 1:2,500 in pediatric populations the focus of this study. Although there is evidence of a familial association of EoE, EoE as with other food allergic and allergic diseases is likely mediated by interaction of environmental allergens (such as foods) with several genes. Food antigens play an essential role in EoE since specific food elimination diets and amino acid formulas are successful as EoE therapy in 60-98% of subjects. Indeed, the majority of children with EoE have specific IgE to foods but they often continue to ingest these foods due to lack of immediate hypersensitivity reactions. This study focuses on the gene-environment interaction of food consumption in food sensitized children with EoE and the role of TGFb1 gene polymorphisms. We hypothesize that in EoE there is a gene polymorphism (TGFb1) environment (food) interaction that contributes to increased IgE mediated TGFb1 expression in the esophagus and increased esophageal remodeling in a subset of EoE subjects. As esophageal stricture formation is an important complication of remodeling in EoE (6-12% of children; 33% of adults), identifying genetic polymorphisms in TGFb1 in EoE may allow the early identification of food sensitized children at risk for the development of this significant complication of EoE. The TGF-b1 gene promoter has a well characterized functional SNP C-509T which results in three genotypes, i.e. TT, CT, or CC. Prior studies have demonstrated that the TT genotype of the TGF-b1 gene is associated with increased levels of fibrosis in progressive kidney disease. We will examine whether the TT genotype is associated with increased fibrosis in food sensitized EE subjects. We hypothesize that the TGF-b TT genotype is associated with increased levels of TGF-b expression in the esophagus in food sensitized EE and promotes the development of esophageal remodeling.

**Body:**

This proposal outlines 6 tasks that were successfully completed during the three year proposal. Task 1 (approval of human subjects) was completed in year 1 of this proposal. In year 1-3 we have successfully completed the remaining tasks 2-6 as outlined below.

**Task 1: Approval for human subjects studies (month 1-6)**

UCSD IRB approval of the DOD modified consents was obtained on April-5-2011.

We received an approval letter from DOD permitting us to start working on the project on April 13, 2011 in an e-mail from Ms Duchesneau (Chief, Human Subjects Protection Review; [Caryn.Duchesneau@us.army.mil](mailto:Caryn.Duchesneau@us.army.mil)).

**Task 2: Enrolling EE (Food IgE+ and Food IgE-) subjects (n= 400 subjects) (month 1-33)****2a) Database for EE genotyping clinical trial established (month 1)**

Our goal was to have >400 subjects in total in the clinical database which we have exceeded with a current total number of >700 subjects in the continuously accruing database. We have genotyped 200 subjects during this grant period, meeting the goal of 60 subjects per year over 3 years. 35% were genotype CC, 53% were genotype CT, 12 were genotype TT.

Overall, 69% of EE subjects are Food IgE+ and 31% are Food IgE- (**Table 1**). The data for the analysis of food IgE sensitization in the context of genotype over the 3 year period is presented below and represents the cumulative data for the entire study.

**2b) Demographic and clinical information entered (month 1-33)**

Demographic and clinical information for all genotyped subjects has been entered into the database on a weekly basis. Similar data is available from previously entered subjects. The overall analysis shows that, consistent with past reports, the majority of our EE subjects are male, Caucasian, and have another atopic disorder (asthma, allergy, eczema and/or food allergy) (**Tables 1, 2**). Overall, 69% of EE subjects are Food IgE+ and 31% are Food IgE- (**Table 1**). 52% of children were skin prick test (SPT) + to foods. In the Food IgE+ group 20-45% have positive IgE to milk, wheat, egg, and/or soy (**Table 3**) with food specific serum IgE elevated in the range of 2-4 kU/L (normal < 0.35) among those subjects who have had serum IgE testing completed (**Table 4**).

**Table 1: Demographic and Food Allergic Characteristics of Pediatric EoE Population**

Age in years	Male (%)	Caucasian	Food IgE* Positive	Food IgE Negative
6.9 years	80	79	69%	31%

\*Serum or skin prick testing positive

**Table 2: Co-existent Allergic Characteristics of Pediatric EoE Population**

Asthma (%)	Allergic Rhinitis (%)	Eczema (%)	Food Allergy (clinical food reaction)
36%	53%	43%	42%

**Table 3: Food IgE Sensitization Pattern**

Food Antigen	IgE Positive (%)
Milk	42
Egg	45
Wheat	29
Soy	20

\*IgE positive is defined as food serum IgE >0.35, or food skin prick test wheal/flare >3/5mm as compared to negative saline control

**Table 4: Levels of serum IgE to Foods**

Serum IgE, mean ku/L			
Egg (n=66)	Milk (n=74)	Wheat (n=68)	Soy (n=62)
4.1	2.7	3.3	3.1

Normal IgE < 0.35; n= number of study subjects with positive serum IgE

## 2c) Results of upper GI endoscopy entered (month 1-33)

Upper GI endoscopy results were captured weekly.

## 2d) Results of esophageal biopsy eosinophils/hpf entered (month 1-33)

Pathology reports are generated in 2 days following the endoscopy with biopsy and results were entered into the database on a weekly ongoing basis (see **Figures** below for distribution by genotype) over each year.

## **Task 3: Quantitating esophageal expression of TGF- $\beta$ and pSmad in EE (Food IgE+ and Food IgE-)**

**Relevant to all tasks:** We have evaluated all subjects who consented to genetics and who had samples with adequate tissue to evaluate remodeling. One hundred, forty-two subjects had adequate tissue for at least one (or more) stains in the lamina propria (LP)

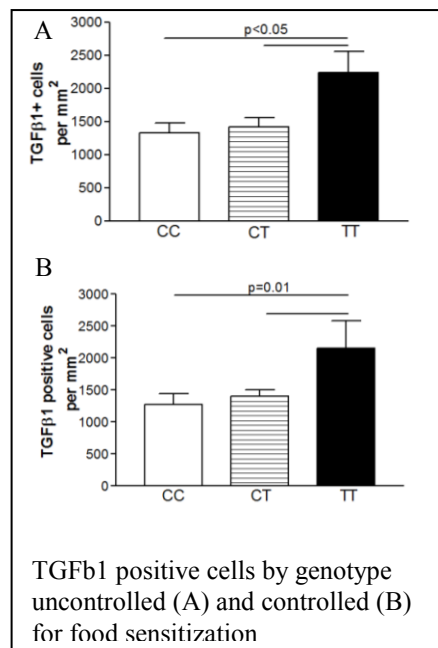
### 3a) Making pathology blocks of esophageal biopsy (month 1-33)

Esophageal biopsy blocks were made following each endoscopy and biopsy and the results of esophageal epithelial and subepithelial findings in the context of remodeling, genotype, and food sensitization follow below.

### 3b) Sectioning esophageal biopsy blocks (month 1-33)

Sectioning of esophageal biopsy blocks was done in an ongoing manner. Sections are first evaluated for the presence of lamina propria (LP) and those with LP are preferentially further evaluated. We have subsequently evaluated remodeling and histologic features all of our genotyped patients who had adequate tissue.

### 3c) TGFβ1 immunostain: To quantitate TGFβ1+ cells (month 1-33)

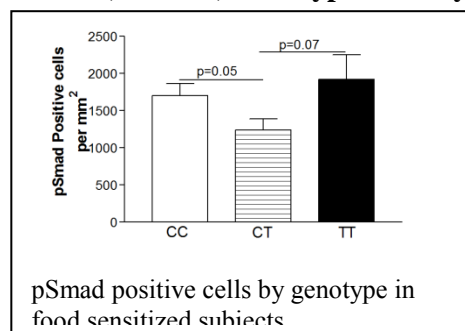


**Figure 1.**

### 3d) pSmad immunostain : To quantitate pSmad+ cells (month 1-33)

Immunohistochemistry and quantitation has been completed on all genotyped subjects and the analysis by food sensitization has been completed. Genotype at C-508T did not influence the numbers of Smad2/3 positive cells.

### Task 3d) and 4d) Genotype-Phenotype for pSmad+ cells in food IgE+ (Figure 2).



in EoE.

**Figure 2.**

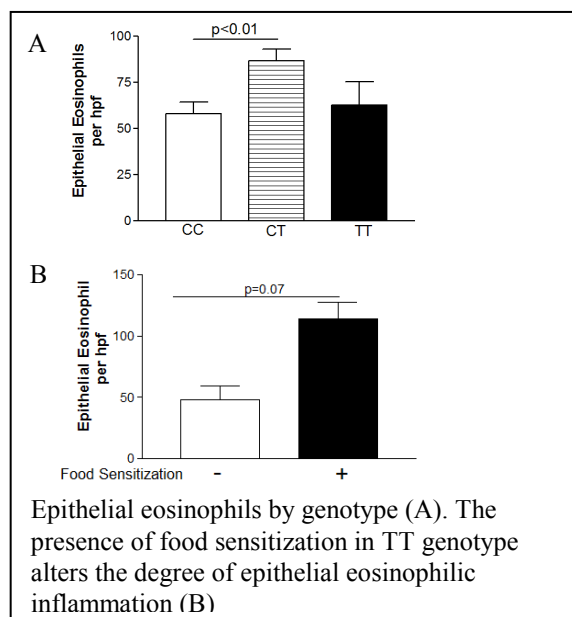
Quantitation of TGFβ1 cells: Immunohistochemistry and quantitation has been completed on all genotyped subjects who had adequate tissue for remodeling analysis. Importantly, we have confirmed that genotype SNP influences the numbers of TGFβ1 positive cells in the esophagus.

**Task 3c) and Task 4d) TGFβ1 genotype-phenotype in the context of IgE+ (Figure 1)** The presence of food sensitization increases the statistical significance of the difference in the numbers of TGFβ1 positive cells in TT as compared to the other 2 genotypes. The presence of food sensitization and a single T allele (that is, TT or CT versus CC) did not influence the numbers of TGFβ1 positive cells. From these data we make the conclusions that TGFβ1 promoter SNP C-509T modifies disease severity as defined by TGFβ1 expressing cells and that the presence of food sensitization does not necessarily further influence this parameter.

had a trend towards influencing the numbers of Smad2/3 positive cells by genotype but not in the manner that we had predicted. CC subjects who were food sensitized had increased pSmad+ cells as compared with CT subjects. Food IgE+ TT subjects had a trend toward increased pSmad+ cells as compared with food IgE+ CT but not TT subjects (p=0.07). Within a single genotype (CC vs CT vs TT) there were no differences in the numbers of pSmad2/3 positive cells when food sensitized versus not food sensitized. From these data, we conclude that food sensitization likely does not significantly influence the numbers of pSmad2/3 positive cells

### 3e) MBP Ab; Immunostain to quantitate eosinophils (month 1-33)

#### 3e.1 Epithelial eosinophils (Figure 3)



As was noted in the last progress report, evaluation of MBP correlated well with the numbers of eosinophils seen on H&E ( $r=0.81$ ,  $p<0.0001$ ) and as such H&E is a complementary method for the numbers of MBP positive cells. All genotyped subjects have been evaluated for the numbers of epithelial and LP eosinophils in the context of food sensitization. These data show that epithelial eosinophils are elevated in CT genotype as compared with CC genotype. This remains the case in food sensitized subjects. Interestingly, food sensitization may influence the level of epithelial eosinophils such that among TT subjects (but not CC or CT genotype subjects) there is a trend toward higher levels of eosinophils in the group of subjects that are food sensitized ( $p=0.07$ ).

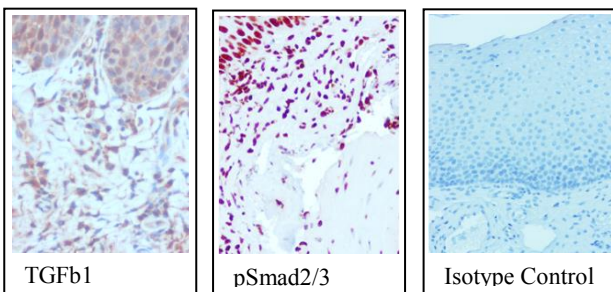
3e.2 We have completed the evaluation of LP eosinophils by food sensitization and genotype. These data show that there are no significant differences in LP eosinophils by

genotype or within genotype with food sensitization.

**Figure 3.**

### 3f) Control Abs (month 1-33)

Control Abs (Figure 4) are routinely used to immunostain slides to exclude non-specific staining of tissues. We have not noted any non-specific staining with either the control antibodies for any of the specific antibodies used in these studies. Representative images for TGFb1, pSmad2/3, and isotype control are shown below



**Figure 4**

### 3g) Slides (month 1-33)

Esophageal tissue sections were sectioned onto slides on a routine basis.

### 3h) Results of esophageal biopsy histology (task 3a-g) entered into the database.

Esophageal histology results were entered into the database on a weekly basis.

#### **Task 4: TGF- $\beta$ genotyping in EE (month 1-33)**

##### **4a) Consent for genotyping in EE subjects (month 1-33)**

200 EoE subjects were genotyped and all of their data is presented as above.

##### **4b) TGF- $\beta$ genotyping (month 1-33)**

All of the subjects who came for routine blood draw have had genotyping completed if consent was in place

##### **4c) TGF- $\beta$ single nucleotide polymorphisms (SNP) information entered into database (month 1-33)**

SNP information was entered into the database on an ongoing basis

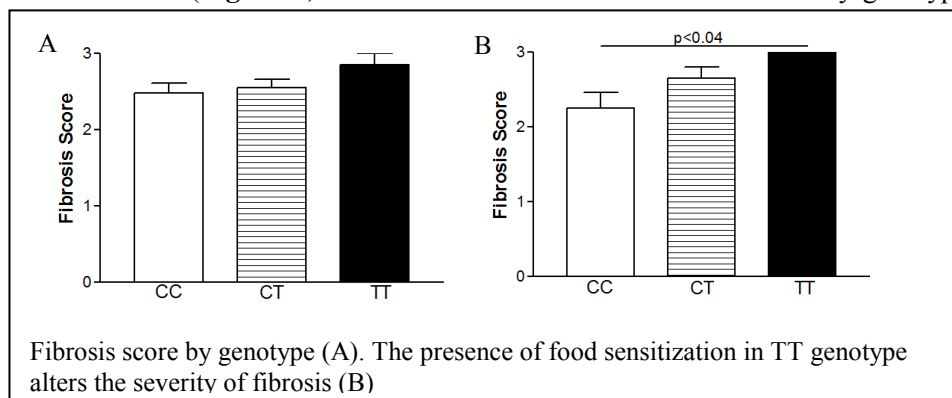
##### **4d) Results of TGF- $\beta$ SNP genotyping, phenotyping, and TGF- $\beta$ /pSmad analysed**

Analysis is complete. See above for TGF $\beta$ 1+ (**Figure 1**) and pSmad+ (**Figure 2**) data and below for LP eosinophils.

#### **Task 5: Quantitating esophageal remodeling in EoE (Food IgE+ and Food IgE-) (month 28-33)**

##### **5a) Trichrome stain: To quantitate fibrosis**

As noted in the last progress reports, we have found H&E to be equivalent to trichrome for assessing our fibrosis score. We have now completed our analysis of fibrosis among the EoE subjects with adequate LP for evaluation (**Figure 5**). We see a trend toward elevated fibrosis by genotype but there is no statistical significance



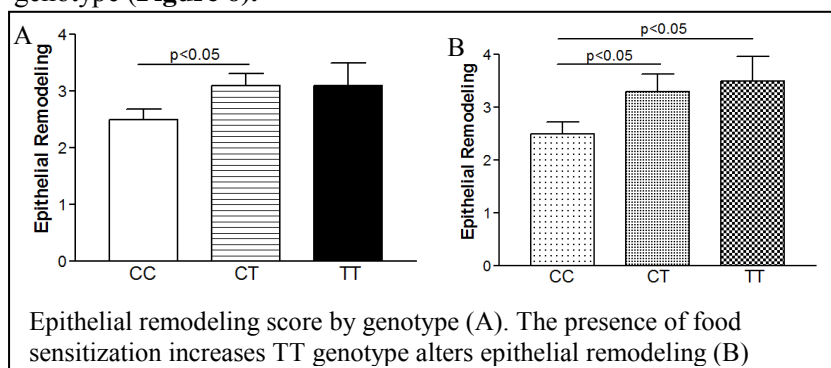
Interestingly, food skin prick positivity to foods modifies this effect such that statistical significance is achieved between the TT and CC subject groups. As such, it seems likely that food sensitization contributes to a more severe histologic phenotype in

terms of fibrosis in the context of TT genotype at C-509T.

**Figure 5.**

##### **5b) H and E stain: To quantitate basal zone hyperplasia (month 28-33)**

Quantitation of basal zone hyperplasia and epithelial remodeling score has been completed in all genotyped subjects. Epithelial remodeling score is derived using our standardized histology scoring tool and consists of the severity of basal zone hyperplasia (scored 1-3) + presence/absence of dilated intracellular spaces (scored 0 or 1) + absence of epithelial desquamation (scored 0 or 1) for a maximum score of 5. CT genotype subjects have significantly higher epithelial remodeling scores as compared with CC subjects. In contrast, TT subjects have statistically higher epithelial remodeling scores only when there is food IgE+. As such, it appears that food sensitization influences the degree of epithelial remodeling in the context of C-509T genotype (**Figure 6**).

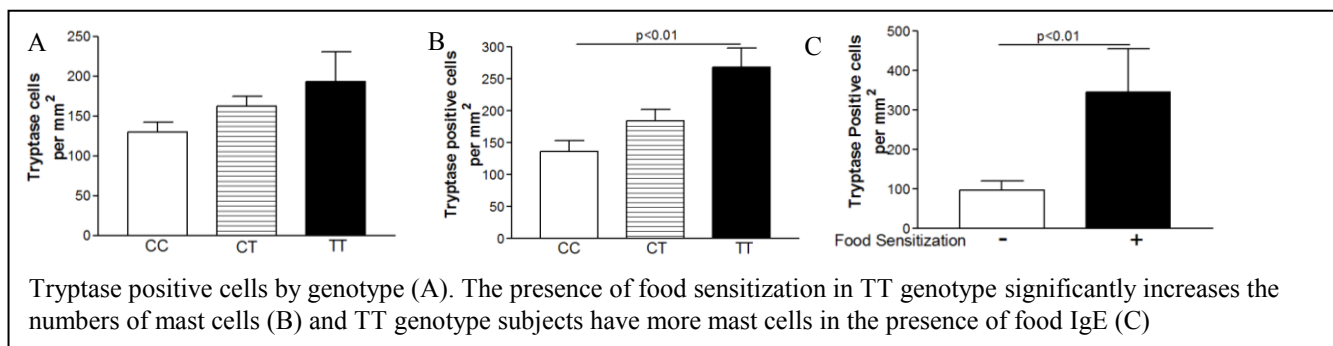


**Figure 6.**



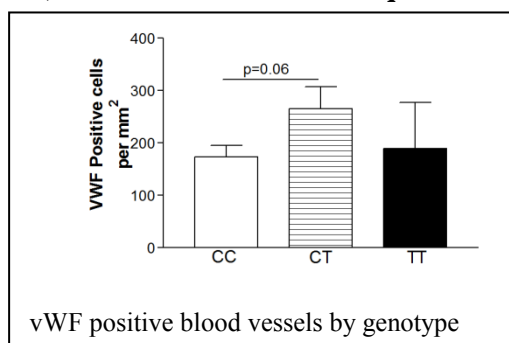
### Tryptase Positive Mast Cells

We have completed tryptase stains on all genotyped subjects and completed the analysis by food sensitization. These results show that there is no difference in tryptase positive cells by genotype (**Figure 7**). However, food sensitization influences the number of mast cells by genotype with TT subjects who are food sensitized demonstrating significantly elevated numbers of mast cells as opposed to CC or CT food sensitized subjects. In addition, food sensitized TT, but not CC or CT, genotype subjects have increased mast cells as compared with TT subjects who are not food IgE+.



**Figure 7**

### 5c) VWF Immunostain: To quantitate blood vessels (month 28-33) (Figure 8)



We have completed vWF staining in all the genotyped subjects. There is a trend toward significance between the CC and CT groups in the presence of food sensitization (p=0.06) but when controlling for the presence of a T allele (CC vs CT+TT), this trend becomes less significant. There were no differences in the numbers of vessels in the presence of food sensitization in any of the genotypes.

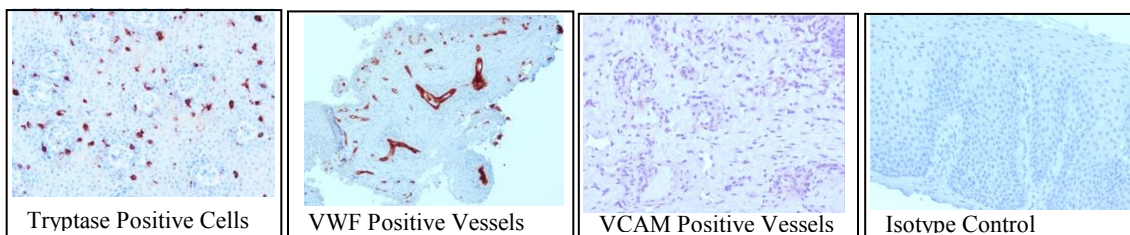
**Figure 8**

### 5d) VCAM Immunostain: To quantitate activation of blood vessels (month 28-33)

We have completed our staining of VCAM on all of the genotyped subjects. There are no differences in VCAM positive vessels by genotype and no influence of food sensitization in any of the genotypes. In addition, within a single genotype, there were no differences in the numbers of VCAM positive vessels by food sensitization.

### 5e) Control abs (month 28-33)( Figure 9)

Control Abs are used to immunostain slides to exclude non-specific staining of tissues. We do not see nonspecific stain in these section. Representative images of isotype control, tryptase, vWF, and VCAM are shown below



**Figure 9**

**5f) Slides (month 28-33)**

Esophageal tissue sections were sectioned onto slides on a routine basis

**Task 6: Preparation of manuscript (month 33-36)**

Draft manuscript is attached.

**Key Research Accomplishments:**

- 200 EoE subjects genotyped
- Continued enrollment in the database that currently totals >700 subjects
- Evaluation of the gene-environment interaction of food-TGFβ1 functional promoter SNP C-509T in 142 subjects in the context of multiple features of remodeling including fibrosis, TGFβ1 expression, pSmad expression, angiogenesis and vascular activation, epithelial remodeling, and epithelial and LP eosinophilic and mast cell inflammation
- Final data that shows the following:
  - Functional TGFβ1 promoter SNP genotype TT associates with higher numbers of TGFβ1 positive cells
  - TGFβ1 promoter SNP genotype TT has significantly more fibrosis only when there is food IgE+
  - Tryptase positive cells (mast cells) are significantly higher in TT only when food IgE+
  - Epithelial eosinophilic inflammation is higher in TT genotype only when there is food IgE+
  - Epithelial remodeling is more severe in TT genotype than CC genotype when there is food IgE+
- Overall, our key research accomplishment is proof of the central hypothesis that the severity of 1) remodeling in the epithelium and LP and 2) inflammation is influenced by the combination of genotype at TGFβ1 C-509T and the presence of food-positive IgE. This is the first data to suggest the presence of a gene-environment interaction in EoE. These data have potentially significant clinical impacts for EoE management as well providing hypothesis generating data for further mechanistic studies in pediatric and adult EoE.

**Reportable Outcomes:**

Preliminary results were reported in oral abstract form at the 2013 AAAAI meeting. We have included a draft of a manuscript in preparation documenting the findings summarized in this final progress report. All of the data summarized above and in the Conclusions below are reportable outcomes.

**Conclusions:**

Our data demonstrate a positive interaction of the environment “food” consumed in EoE subjects and the severity of their disease. Since the major complications of food impactions and stricture are mechanistically embedded in remodeling, an understanding of EoE pathogenesis in the context of disease modifying genes is of utmost importance. EoE is a chronic disease, progressing essentially uniformly to strictures when untreated. In this context, the removal of foods may be essential to successful EoE management in certain patient phenotypes.

In this study, we demonstrate a number of novel findings in EoE and report the first gene-environment interaction study in this disease. Firstly, we find that the number of TGFβ1 expressing cells is highest in the SNP variant TT that creates a binding site for the transcription factor YY-1 and which leads to increased TGFβ1 production. Since our prior studies have demonstrated that both eosinophils and mast cells make TGFβ1 in EoE subjects, we would predict that these are the cellular compartments responsible for this increase. Indeed, there is a trend toward significant differences in the numbers of epithelial eosinophils in TT patients when they are food sensitized. In addition, among CC, CT, and TT subjects, there are increased numbers of mast cells in those subjects who have TT genotype and food sensitization and TT subjects with food IgE+ have more mast cells than TT subjects who are food IgE-. Epithelial remodeling is significantly worse in the presence of food sensitization in genotype TT as compared with CC genotype. We further show that the stepwise gradient in fibrotic severity from CC to CT to TT is statistically significant only when controlled for subjects who have SPT positivity to foods.

Together these data suggest a pathogenic mechanism in EoE whereby pro-fibrotic factor release by inflammatory cells could be mediated by the consumption of a food to which a subject is sensitized. Unlike IgE mediated allergy, subjects with EoE continue to consume foods to which they are sensitized due to a lack of immediate reactions that warn the subject that they are having a food reaction. In this context, the environment of food exposure to which the EoE patient is sensitized could drive local mast cell degranulation. Since subjects with TT genotype have increased numbers of mast cells, this genotype may be more prone to such effects. Release of mast cell mediators could have multiple effects, including the recruitment or maintenance of eosinophils via cytokines such as IL-5 and fibrosis and smooth muscle dysfunction via the production of TGFβ1. This release of pro-fibrotic factors such as TGFβ1 could predispose subjects to higher fibrosis and faster rate to stricture and/or higher rates of food impaction. As such, our data suggest that strategies that include the avoidance of foods to which a subject is sensitized may be a relevant therapeutic intervention, especially in TT genotype subjects.

**References:**

None.

**Appendices:**

Abstract Attached (Anilkumar et al; 2013)

Draft manuscript attached (Anilkumar et al; 2014)

**Manuscripts/Reprints, Abstracts:**Abstract:

Anilkumar AA, Newbury RO, Dohil R, Mueller J, Hoffman HM, **Broide DH, Aceves SS**. A Transforming Growth Factor Beta-1 Gene Single Nucleotide Polymorphism May Influence Phenotype in Pediatric Eosinophilic Esophagitis. *J Allergy Clin Immunol*;131:S132. 2013. Presented as an oral abstract in at AAAAI meeting in March 2013

Anilkumar AA, Newbury RO, Rawson R, Palmquist J, Yang T, Aquino M, Hoffman HM, Mueller J, Dohil R, **Broide DH, Aceves SS**. A gene-environment interaction between food and TGFβ1 promoter SNP C-509T in pediatric eosinophilic esophagitis. Manuscript in preparation. 2014

## **A transforming growth factor beta-1 gene single nucleotide polymorphism may influence phenotype in pediatric eosinophilic esophagitis**

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### **Rationale:**

Eosinophilic esophagitis (EoE) is a chronic antigen driven disease associated with tissue remodeling and increased TGFb1 expression. The genetic influences on disease severity and therapeutic response are currently unknown. A functional single nucleotide polymorphism (SNP), C-509T, in the TGFβ1 gene promoter has been linked to asthma and renal disease severity. We hypothesized that this SNP may influence histologic and/or phenotypic findings in pediatric EoE.

### **Methods:**

We performed single nucleotide polymorphism analysis for TGFβ1 C-509T in pediatric EoE subjects. Histology scores were generated by a pathologist blinded to genotype and clinical course. TGFb1+ cells were quantitated using immunohistochemistry and image analysis. Subject response to topical corticosteroids was evaluated in the context of genotype.

### **Results:**

Of 129 EoE subjects, 41 (32%), 75 (58%), and 13 (10%) were genotype CC, CT, and TT, respectively. 63 subjects had adequate lamina propria (LP) for analysis. Subjects with genotype TT had significantly more TGFβ+ cells (mean=2511cells/mm<sup>2</sup>, SEM =150) than genotypes CT (1532cells/mm<sup>2</sup>, SEM=64) or CC (mean=997cells/mm<sup>2</sup>, SEM=90) ( $p < .0001$ ). TT subjects had higher numbers of LP eosinophils (20/ hpf versus CT-13/hpf and CC -12/hpf) while CC subjects had lower numbers of peak epithelial eosinophils (mean=57.9 per hpf) than CT (100.2/hpf) or TT (66.7/hpf) subjects ( $p<0.001$ ). 49 subjects were treated with topical esophageal corticosteroids (TCS). While 94% of CC subjects responded to TCS, CT and TT subjects had more variable response rates of 73% and 57%, respectively (OR=4.6,  $p=0.08$ ).

**Conclusion:** The functional C-509T SNP in the TGFβ1 gene may influence EoE histology and phenotype.

**Funding Sources:** DOD. NIH

A gene-environment interaction between food and TGF $\beta$ 1 promoter SNP C-509T in pediatric eosinophilic esophagitis

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Conflicts: The authors have no relevant conflicts of interest to declare

## Abstract

**Background:** Eosinophilic esophagitis (EoE) is a chronic antigen mediated disease associated with substantial esophageal remodeling and fibrosis. The functional promoter SNP in the TGFb1 promoter C-509T associates with renal fibrosis and asthma as well as increased numbers of TGFb1 expressing cells in the EoE esophagus. Like other allergic diseases, EoE is likely to be polygenic and have gene-environment interactions. We conducted a study to understand the relationship between EoE disease severity, TGFb1 C-508T SNP, and food sensitization.

**Methods:** Genotype at the TGFb1 promoter SNP C-509T was analyzed in 200 subjects with EoE. Subject phenotype for IgE sensitization to the disease relevant antigens milk, egg, soy and/or wheat was analyzed using serum food specific IgE or skin prick testing (SPT). The degree of esophageal remodeling was analyzed using a histology scoring tool and immunohistochemistry with quantitation for TGFb1- and pSmad-positive cells, as blood vessels and vascular activation and analyzed in the context of food specific IgE positivity.

**Results:** Of the 200 subjects, 35%, 53%, and 12%, were genotype CC, CT, and TT, respectively at C-509T. Of these 200 subjects, 142 had adequate tissue for an analysis of esophageal remodeling. Among the subjects, 42%, 45%, 29%, and 20% had food sensitization to milk, egg, wheat, and soy, respectively. CC genotype subjects had the lowest levels of TGFb1-positive cells (1313 per mm<sup>2</sup>) while TT subjects had the highest levels (2239 per mm<sup>2</sup>) ( $p<0.05$ ) and this was not affected by the presence of IgE+ to these foods. By comparison, the degree of lamina propria fibrosis ( $p<0.05$ ), the severity of epithelial remodeling ( $p<0.05$ ), and the numbers of epithelial tryptase positive cells ( $p<0.01$ ) were all elevated in EoE subjects with TT genotype (as compared with genotype CT or TT) only when food sensitization was concurrently present. Epithelial mast cells ( $p<0.01$ ) and eosinophils ( $p=0.07$ ) were higher in TT genotype subjects when food sensitization was present. There were no differences in LP eosinophils, angiogenesis, or vascular activation by genotype in the presence or absence of food sensitization.

**Conclusions:** Our data suggest a gene-environment interaction between food IgE+ and the TGFb1 SNP C-509T in EoE subjects. Since EoE subjects often continue to consume foods to which they are sensitized, these findings may have clinical impacts for disease management.

**Key Words:** Eosinophilic esophagitis, Remodeling, Food specific IgE, Food sensitization, Food allergy, C-509T Transforming Growth Factor- $\beta$ 1, Fibrosis

## Introduction

Eosinophilic esophagitis (EoE) is a chronic, largely food antigen mediated disease of increasing worldwide prevalence. Complications include esophageal rigidity and dysmotility with resultant dysphagia, food impactions, and strictures. Esophageal remodeling leads to fibrosis, angiogenesis, and smooth muscle changes and is believed to be the underlying mechanism for disease complications. The risk factors, reversibility, and rate of progression for esophageal remodeling are not entirely clear but these issues are of significant clinical importance if we hope to halt or reverse remodeling.

The majority of untreated adult EoE subjects progress to esophageal narrowing. In adults, increased duration of untreated disease and decreased use topical esophageal corticosteroids are both risk factors for strictures and food impactions. While adult studies show that remodeling is reversible in a subset of subjects, this does not appear to uniformly be the case. Children who are successfully treated with topical corticosteroids and/or elimination diet have clear reversal in esophageal remodeling when they have an epithelial histologic response to therapy. Unfortunately, not all children or adults respond to either dietary elimination or topical corticosteroids. It is likely that there are genetic mechanisms for the variations in therapeutic responses. This has been suggested by a limited number of studies in pediatric EoE showing that subjects who respond to topical esophageal fluticasone have FKBP51 up-regulation. In addition, we have previously shown that children who have a CC genotype at the functional TGFb1 promoter SNP C-509T are more likely than CT or TT genotype subjects to respond to oral viscous budesonide.

The majority of pediatric subjects with EoE have food sensitization with low levels of serum IgE to foods. However, the clinical implication of this observation is not clear since EoE subjects often do not have anaphylaxis to the foods to which they are sensitized. In addition, isolated utilizing skin prick positive foods is not successful for creating an elimination diet in children or adult EoE subjects. It is not clear if the presence of food specific IgE associates with more severe EoE, altered disease course, or local mast cell degranulation although one study suggested that EoE subjects with positive IgE testing had more esophageal mast cells.

We have previously reported that children with genotype TT at the functional TGFb1 promoter SNP C-509T have increased numbers of TGFb1 positive cells in the EoE esophagus. We have also previously reported that subjects with a T allele (CT or TT) have a lower likelihood of response to topical esophageal corticosteroids than CC genotype subjects. Although this implies that CT/TT subjects have more severe EoE in terms of histology, remodeling, and therapeutic response, this has not been proven. In addition, the interaction of environmental factors and genotype at C-509T in EoE has not been investigated. However, current literature

demonstrates that TGFb1 C-509T influences disease severity and IgE production in other atopic diseases such as asthma.

Based on these observations, we hypothesized that there may be a gene-environment interaction in EoE with foods consumed being the “environment” and the TGFb1 C-509T SNP being the “gene”. Since EoE subjects consume foods to which they are sensitized and since C-509T can align with TGFb1 production, and possibly disease severity, we hypothesized that food sensitized EoE subjects with genotype CC, CT, and TT would have different EoE phenotypes in terms of TGFb1 production and disease severity. We demonstrate here the first study undertaken in EoE to understand the relationship between an environmental trigger, food, and genotype.



## Methods

### *EoE subjects/biopsies*

EoE subjects treated during routine clinical care and upper endoscopy (esophagogastroduodenoscopy, EGD) with biopsy at the UCSD/RCHSD eosinophilic gastrointestinal disorders clinic were recruited for genetics and database studies. EoE was defined as EoE  $\geq 15$  eosinophils per high power field (hpf) on hematoxylin/eosin (H&E) stain at 400x magnification on light microscopy in the presence of typical symptom and endoscopic features. Serum or skin prick testing (SPT) for foods was performed as part of routine clinical care. Positive serum testing was defined as  $>0.35$  kU/L. Positive SPT was defined as 3mm wheal and 5mm flare larger than the saline control. Paraffin embedded biopsy specimens were analyzed for the presence of adequate LP for analysis (defined as at least 3 hpf). A total of 200 subjects were genotyped and 142 biopsies had adequate LP for remodeling analysis. All studies were approved under UCSD/RCHSD IRB numbers 091485 and 081415.

### *Immunostaining and histologic assessment*

H&E stained, formalin fixed, paraffin embedded specimens were evaluated by a single pathologist blinded to the diagnosis and treatment (RN). The numbers of epithelial and lamina propria (LP) eosinophils, the severity of basal zone hyperplasia, and the LP fibrosis score were quantified using our previously published pathology scoring tool.<sup>16</sup>

Tissue sections (5 $\mu$ M) were deparaffanized and hydrated prior to immunostaining as previously described.<sup>2</sup> Species appropriate secondary antibodies and AEC or DAB were used for immunohistochemistry.<sup>2</sup> The mean of the peak numbers of TGF $\beta$ 1 and pSmad2/3 in 3 hpf in the LP are reported. The mean peak numbers of tryptase positive cells in 3 hpf the epithelium is reported. The mean peak numbers of vessels staining positively for von Willebrand's Factor (vWF) and vascular cell adhesion molecule-1 (VCAM-1) in the LP are reported. All images were quantified and analyzed under identical light or fluorescence microscopic conditions, including magnification, gain, camera position, and background illumination.

### *Analysis of the C-509T SNP of the TGF $\beta$ 1 promoter*

Patient's DNA was isolated from peripheral blood and the C-509T SNP of the TGF $\beta$ 1 promoter was analyzed. The TGF $\beta$ 1 promoter genotype was assessed using forward (5' GGGGACAGTAAAAT-GTATGGG-3') and reverse (5'-GTCACCAGAGA- AAGAGGAC-3') TGF $\beta$ 1 promoter primers followed by digestion with DdeI to yield 242, 189, and 53 base-pair products analyzed by gel electrophoresis. SNP analysis was also performed

using Taqman based PCR analysis for CC versus CT versus TT genotype. Genotype was confirmed in a subset of subjects using direct sequencing. The TGF $\beta$ 1 promoter was sequenced using forward (CAGACTCTAGAGACTGTCAG) and reverse (GTCACCAGAGAAAGAGGAC) primers for PCR and direct sequencing for C-509T SNP genotype. Sequence analysis was performed using Sequencher (Genecodes, Ann Arbor MI).

### *Statistics*

Between group comparisons were done using NCSS and Graphpad Prism statistical software. T-test was used for continuous variable and for data with a normal distribution. Mann-Whitney U test was used for dichotomous variables. A two tailed p value <0.05 was considered statistically significant. Graphs were created using Graphpad Prism.

## Results

### *Clinical characteristics*

Two hundred EoE subjects were genotyped at promoter SNP C-509T. Of these subjects, 35% were genotype CC, 53% were CT, and 12% were TT. The mean age of the subjects was 6.9 years. Eighty percent of the subjects were male and 79% were Caucasian. Consistent with high rates of atopy in the EoE population, 36% had asthma, 53% had allergic rhinitis, 43% had eczema, and 42% had an immediate hypersensitivity reaction to a food reported by the parent.

We assessed food sensitization to the most common EoE food triggers, specifically milk, wheat, egg, and soy. Among these 200 children, 69% had food positive IgE to one or more of these foods by serum or skin prick testing. The most commonly positive food on testing was egg with 45% of the population being skin or serum sensitized to egg white. Milk was the next most common antigen to which the children were sensitized (42%) followed by wheat (29%) and soy (20%). The level of food sensitization in these children was low and would not predict an immediate hypersensitivity response to the food based on published predictive values for milk or egg. The average level of IgE (kU/L) to egg was 4.1, to milk was 2.7, to wheat was 3.3, and to soy was 3.1.

### *Epithelial remodeling and inflammation*

In order to assess the degree of histologic remodeling in the epithelium, we utilized our epithelial remodeling scoring tool. This grades the severity of basal zone hyperplasia from 1-3 based on the percent of the epithelial height that is comprised of basal cells + the presence or absence of dilated intercellular spaces + the presence or absence of epithelial desquamation. CC, CT, and TT genotype subjects had a mean epithelial score of 2.5, 3.1, and 3.1, respectively with a significant difference only between the CT and CC groups ( $p<0.05$ ) prior to assessing for food sensitization (Figure 1a). However, when comparing the groups when food specific IgE was positive, there was a significant difference between the CC and TT (mean score=3.5) ( $p<0.05$ ) as well as a continued significant difference between the CC (mean score=2.5) and CT (mean score=3.3) populations (Figure 1b). As such, the presence of food sensitization associated with more severe epithelial remodeling in TT subjects only when food sensitization was present. This data suggests an interaction between the T allele at C-509T and food sensitization.

The mean number of epithelial eosinophils per hpf were higher in CT genotype (87 per hpf) patients as compared with CC (58 per hpf,  $p<0.01$ ) and TT (63 per hpf,  $p=0.10$ ) (Figure 1c). However, within the TT genotype, there was a statistically non-significant increase in the number of epithelial eosinophils in those

subjects who had food-specific IgE (114 per hpf) as compared with those without food specific IgE (48 per hpf) ( $p=0.07$ ) (Figure 1d).

We also assessed the numbers of tryptase positive cells in the epithelium of these EoE subjects by genotype. There was statistically insignificant trend towards higher mast cell numbers from genotype CC to CT to TT overall (Figure 2a). Interestingly, however, when assessing only those children who had food specific IgE, TT genotype subjects had statistically higher numbers of mast cells in the epithelium (268 per  $\text{mm}^2$ ) than those subjects who were genotype CC (136 per  $\text{mm}^2$ ) or CT (184 per  $\text{mm}^2$ ) (Figure 2b,c). In addition, only within the TT group, there were an increased number of tryptase positive cells when food sensitization was present (346 versus 97 per  $\text{mm}^2$ ) (Figure 2d). This difference was not seen within the CC or CT groups.

#### *Lamina propria remodeling and inflammation*

We assessed LP features by genotype in these subjects. The numbers of TGF $\beta$ 1 expressing cells in the LP were higher among subjects who were genotype TT (2239 cells per  $\text{mm}^2$ ) as compared with subjects who were genotype CC (1313 cells per  $\text{mm}^2$ ) or CT (1422 cells per  $\text{mm}^2$ ) ( $p<0.05$ ) (Figure 3a, c). When assessing the numbers of TGF $\beta$ 1 positive cells among only those subjects who had food specific IgE, the difference between the TT genotype and other two groups became more statistically significant ( $p<0.01$ , Figure 3b) but the trends did not change in terms of numbers of TGF $\beta$ 1-positive cells.

In order to understand if there were differences in histologic fibrotic severity by genotype, we used our standardized fibrosis score. There was a trend of increasing fibrosis scores from CC (mean=2.5) to CT (mean=2.6) to TT (mean=2.9) genotype (Figure 4a). However, a significant difference between TT and CC subject fibrosis scores was seen only when those children with food positive SPT were assessed by genotype. Food sensitized TT, CT, and CC subjects had mean fibrosis scores of 3.0, 2.7, 2.3, respectively ( $p<0.05$  between CC and TT) (Figure 4b) ( $p<0.05$ ). These data suggest that fibrotic severity in EoE is worse when TT promoter genotype is present.

We also assessed the numbers of the cells expressing the canonical TGF $\beta$ 1 signaling molecule, pSmad2/3. There were no differences in the numbers of pSmad2/3 subjects overall but both CC and TT genotype subjects had more pSmad2/3 positive cells than CT subjects (data not shown). In addition to pSmad2/3 we assessed the numbers of vessels and activated vessels. We did not find any differences in the numbers of vWF or VCAM-1 positive vessels by genotype, even in the presence of food specific IgE (data not shown). Lastly, we quantified the numbers of LP eosinophils and found that these also had no differences by genotype in the absence or presence of food sensitization.

## Discussion

In this study we demonstrate a number of novel findings in pediatric EoE subjects. First, we confirmed our original observation seen in a much smaller cohort of subjects that genotype TT at C-509T associates with increased numbers of TGFb1 expressing cells. This was not changed by the presence or absence of food sensitization. Secondly, we demonstrate that fibrosis is increased in TT genotype patients only when the subjects in all three genotypes are food sensitized, suggesting that there is interplay between the presence of food specific IgE and promoter genotype. Consistent with this observation, we found that significant differences in epithelial remodeling and tryptase positive cells occur between TT and CC genotypes subjects only when there is food specific IgE present to disease relevant antigenic triggers of milk, wheat, egg and/or soy. Interestingly, within the TT genotype, children who were food IgE positive had more mast cells and a trend towards more epithelial eosinophils than those who were food IgE negative. As such, our data together suggests that components of EoE severity depend on the presence of both TGFb1 C-509T SNP and food sensitization.

Children with EoE often continue to consume the foods to which they are sensitized, especially at the time of their diagnosis. Indeed, it is common for pediatric EoE patients to have substantial IgE sensitization. However, although their rate of food anaphylaxis is higher than the general population, they do not have immediate hypersensitivity reaction to many of the foods to which they are sensitized. These observations combined with the fact that IgE based elimination diets are not successful in EoE management has made the role of food specific IgE in EoE unclear. Indeed, the mechanisms underlying a lack of immediate reactions in the presence of food sensitization in EoE remain ambiguous. However, our data suggest that the presence of food specific IgE in the context of certain genetic underpinnings, such as TT genotype at the TGFb1 promoter, could predispose to more a more severe and fibrotic phenotype. It will be interesting to evaluate if those children who are of CT and/or TT genotype and do not respond topical corticosteroids have higher rates of food sensitization. If this is the case, there may be implications for disease management such as the concurrent use of medications and elimination diets for full disease control. Our data may also have further management implications such as a need for more aggressive inflammatory management in the presence of both TT genotype and food sensitization.

Our data is consistent with other data in asthmatic subjects with TT genotype. It has been demonstrated that the TT SNP results in a binding site for the transcription factor, ying-yang-1 (YY-1) thereby driving increased TGFb1 expression. Indeed asthmatic subjects with TT genotype have increased mRNA for TGFb1. This is consistent with both the higher numbers of TGFb1 positive cell as well as the higher fibrosis scores in TT genotype subjects. When this layered with food sensitization, the results are more robust.

Our rates of CC, CT, and TT genotype are consistent with those found in the general population. These data support the notion that TGFb1 C-509T is not a risk allele, but rather, a disease modifying allele in EoE. Interestingly, a recent publication has demonstrated that among asthmatic subjects with genotype TT, there is a higher risk for loss of control of clinical disease. In this context, it will also be interesting to understand if the T allele in EoE associates with those subjects who lose long-term control of their disease despite adherence to prescribed medications and/or diets. This is particularly important in children since EoE is a chronic disease with very high relapse rates upon removal of the disease-controlling therapy.

The role of TGFb1 in allergic diseases is complex. However, it does appear that higher levels of TGFb1 and/or TGFb1 signaling associates with a more severe allergic phenotype. For example, people with Loewy's-Dietz syndrome which is caused by increased signaling through the TGFb1 receptor have higher rates of food allergy. In addition, recent data has shown that connective tissue disorders such as Marfan's syndrome in which there is also increase TGFb1 signaling, associates with EoE.

In conclusion, our current data support a model in which EoE subjects with TT genotype have a baseline state of elevated TGFb1 expressing cells. We would hypothesize that these cells are comprised of eosinophils and mast cells since we have previously demonstrated that both of these cell types express TGFb1 in the EoE esophagus. The presence of food specific IgE in the context of TT genotype could then lead to increased numbers of allergic cells such as eosinophils and mast cells. In the presence of food specific IgE, there would be increased local mast cell degranulation and release of pro-fibrotic molecules such as TGFb1. This in turn would cause increased fibrosis in subjects who concurrently have food sensitization and TT genotype. It will be interesting to verify these findings and to understand the further clinical implications of the TGFb1 promoter C-509T SNP in the pathogenesis of EoE severity and response to therapy.

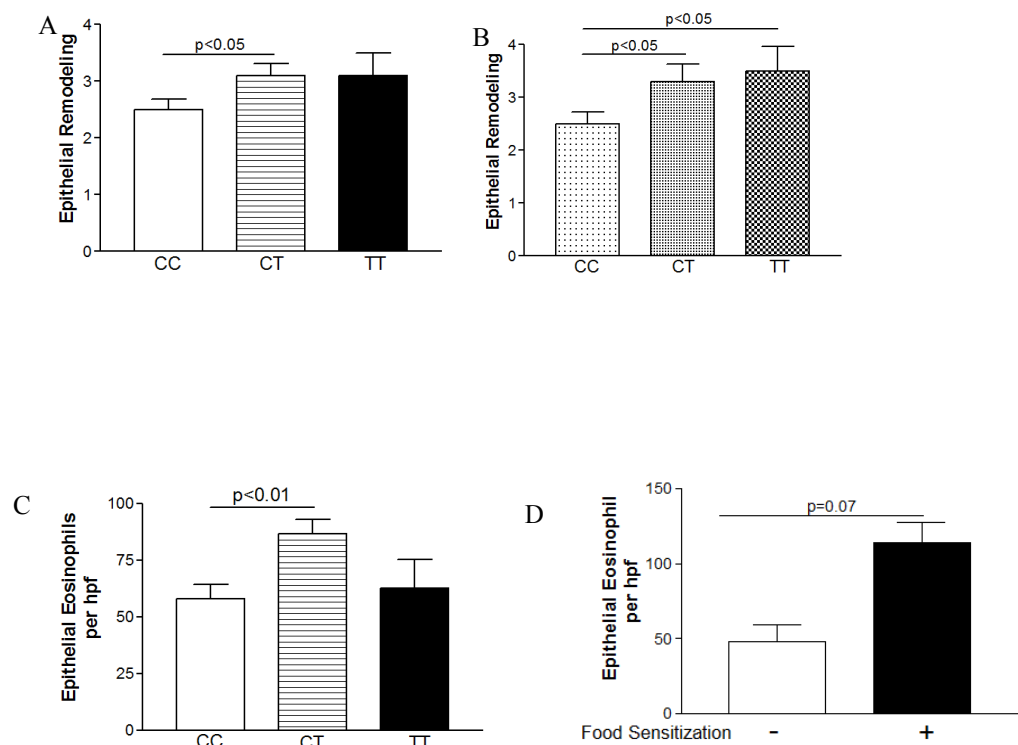
## Figure Legends

**Figure 1.** Epithelial remodeling score by genotype in the entire population (A) and when filtered for those children with food specific IgE by genotype (B). Epithelial eosinophils in the cohort by genotype (C) and among TT genotype subject with and without food specific IgE (D)

**Figure 2.** Tryptase positive cells in the cohort by genotype (A) and in the presence of food specific IgE (B). Tryptase positive cells in TT genotype subjects with and without food specific IgE (C)

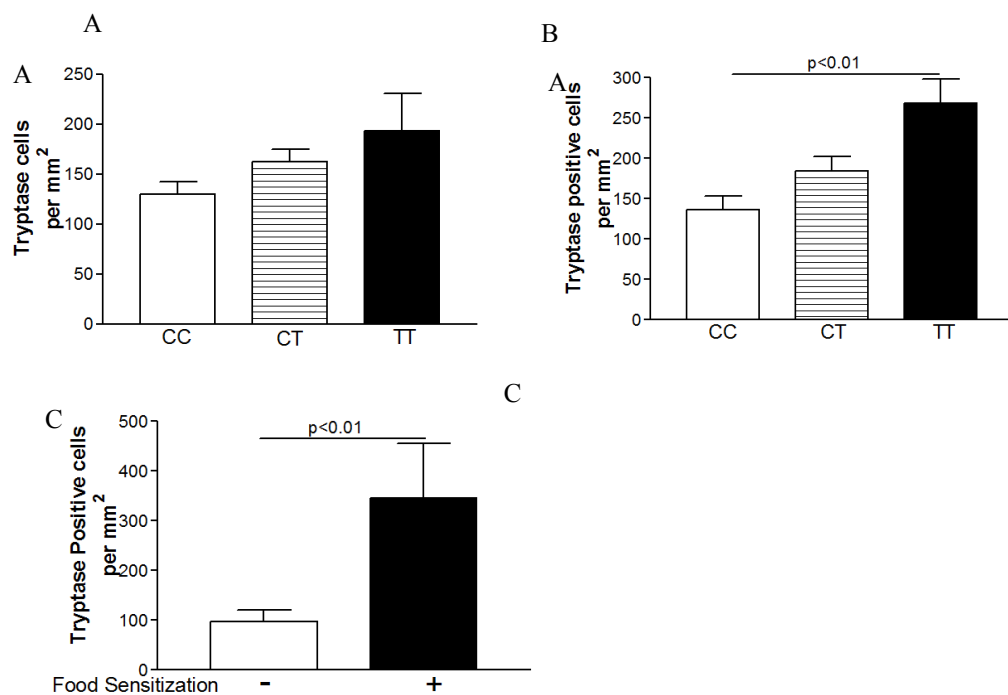
**Figure 3.** TGFb1 positive cells in the cohort (A) and among those who have food specific IgE (B). Representative images (400x) of TGFb1 positive cells in subjects with CC and TT genotypes (C)

**Figure 4.** Fibrosis score by genotype in the cohort (A) and by genotype among those with skin prick test positive results to food (B)

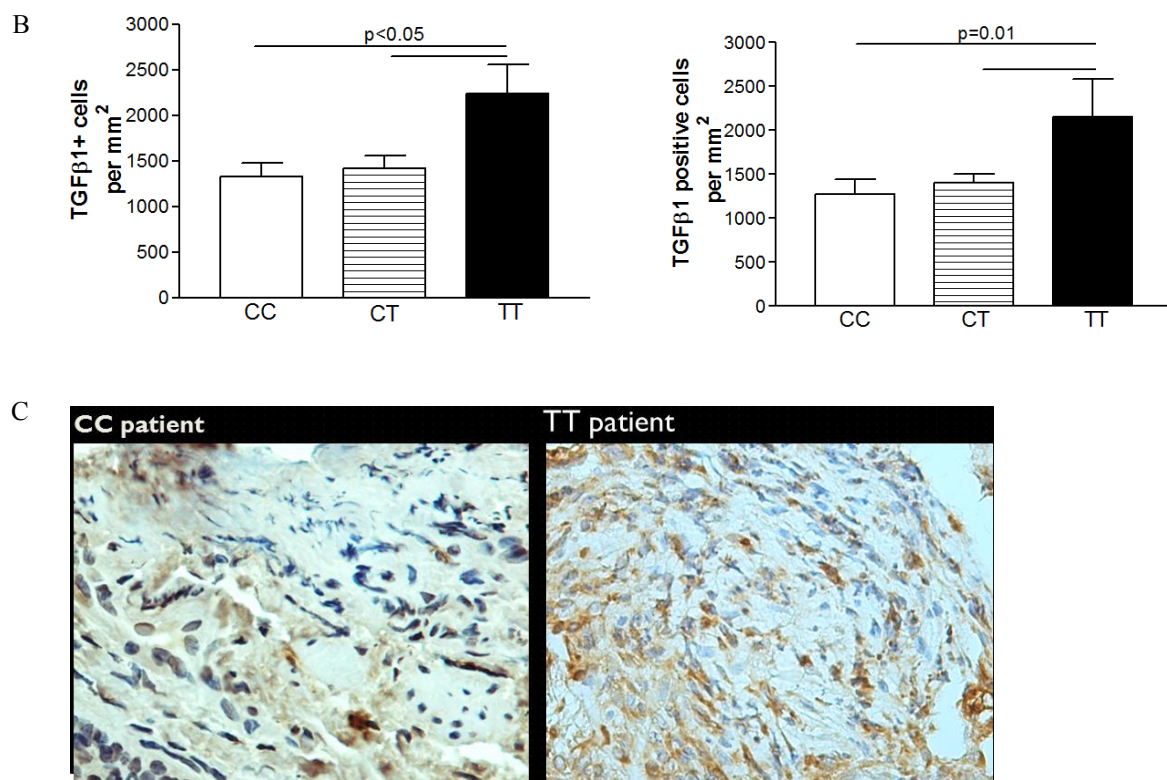


**Figure 1.** Epithelial remodeling scores and eosinophils by genotype and food IgE +

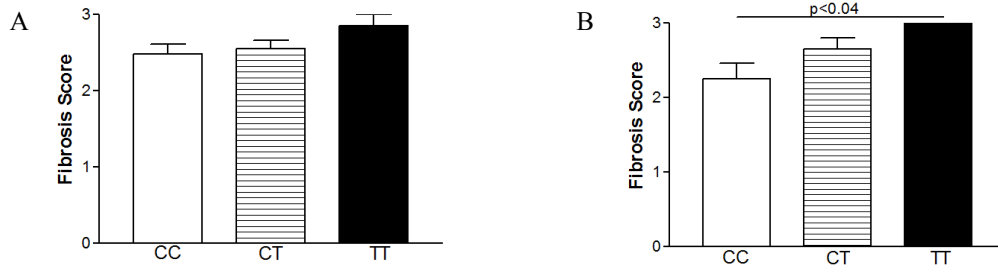




**Figure 2.** Tryptase positive cells by genotype and food specific IgE +



**Figure 3.** TGFβ1 positive cells by genotype and food specific IgE positivity



**Figure 4.** Fibrosis score by genotype and food specific IgE +